

# Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater

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## Abstract

The effects of humic acid (HA) on the toxicity of copper to sea urchin *Paracentrotus lividus* larvae were studied in chemically defined seawater. Square Wave Anodic Stripping Voltammetry (SWASV) was employed to study the complexation of copper in seawater medium. A simple complexation model assuming one ligand type and a 1:1 reaction stoichiometry successfully explained the inverse titration experiments. A conditional stability constant of  $6.53 \pm 0.05$  and a complexating capacity of  $230 \pm 7 \mu\text{mol Cu/g HA}$  were obtained. Sea urchin bioassay tests with two endpoints, embryogenesis success and larval growth were carried out in order to study the toxicity of dissolved copper in both the presence and absence of HA. The toxicity data obtained fitted well into a logistic model, and the high sensitivity of both endpoints ( $\text{EC}_{50}$  were  $41.1 \mu\text{g Cu/l}$  and  $32.9 \mu\text{g Cu/l}$ , respectively) encourages their use for biomonitoring. The HA had a clearly protective effect, reducing the toxicity of Cu to the sea urchin larvae. The labile copper, rather than the total copper concentrations, explained the toxicity of the Cu-HA solutions, and the Cu-HA complexes appeared as non-toxic forms. These results are in agreement with the Free Ion Activity Model, because the labile Cu concentrations in this buffered and chemically defined medium covary with the free ion activity of the Cu, validating the model to naturally occurring HA in the marine environment. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Copper; Humic acid; Speciation; Stripping voltammetry; Toxicity; *Paracentrotus lividus*

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## 1. Introduction

Copper is an essential element for organisms, being present in many enzymes involved in metabolic processes that depend on redox reactions (Scheinberg, 1991). Nevertheless, at high

concentrations copper is highly toxic to many organisms, especially in aquatic systems (Knezovich et al., 1981). Copper is one of the most toxic metals, along with mercury and silver, to marine phytoplankton (Sunda and Guillard, 1976), bivalve larvae (His et al., 1999), echinoderm larvae (Kobayashi, 1981) and crustacean larvae (Young et al., 1979). It is also a metal of environmental concern because its concentrations in some polluted areas reach toxic levels.

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The availability and toxicity of metals to organisms living in the water column depend on the chemical speciation of the metals in that particular aquatic environment (Florence, 1983; Blust et al., 1991; Vercauteren and Blust, 1996). Many metal species show little or no bioavailability, e.g. metals adsorbed on particulate matter, carbonate complexes and metals complexed with strong chelating agents such as EDTA. Morel (1983) proposed that the bioavailability of a dissolved metal is related to its free ion activity. The first step in the uptake of metals by an organism comprises the transport of the cation through the plasmatic membrane. This lipidic membrane is a barrier to the simple diffusion of ions due to its hydrophobic nature, and this transport is usually assumed to be mediated by transmembrane proteins with complexing groups that bind the metal (Simkiss and Taylor, 1995). Morel's Free Ion Activity Model (FIAM) assumes, first, the diffusion of the cation from the bulk solution to the membrane surface and that the kinetics of the reaction between the ligand and the metal are rapid and non-limiting compared with the transport process through the membrane; and secondly, that the carrier is in excess and there is thus no saturation (Morgan and Stumm, 1991; Campbell, 1995; Parker and Pedler, 1997). Taking into account these assumptions and the chemical reactions between the metal ion and the ligands present both in the solution and in the carrier protein, it can be demonstrated that the uptake is related only to the free ion activity of the metal. Nevertheless, changes in the composition and conditions of the medium may modify the structure of the plasmatic membrane and the number of available ligand groups present in the carrier proteins, hence affecting their bioavailability also (Blust et al., 1991, 1992; Campbell, 1995; Van Ginneken et al., 1999). The FIAM has been studied widely by using several metals complexed to both inorganic and organic ligands. Nevertheless, speciation studies using naturally occurring organic matter such as humic material are scarce and have been inconclusive because of the poly-functional properties and ill-defined nature of such matter (Campbell, 1995). Furthermore, there is a lack of data in saltwater, because most of

these studies were performed using Cu measured by means of an ion-specific electrode, and interference with chloride ions does not allow this method to be used in seawater (Mota and Correia dos Santos, 1995).

Humic and fulvic acids are a general category of natural and heterogeneous organic substances, with refractory properties and high molecular weight (Hessen and Tranvik, 1998). In many aquatic systems, they may make up between 60 and 80% of the dissolved organic carbon (Ortego and Benson, 1992), although this percentage is usually lower in marine environments (Ma et al., 1999). Humic substances have carboxylic and phenolic groups that can complex trace metals, especially the intermediate metals, i.e. between A and B cations according to Ahrlund or between hard and soft ions according to Pearson (Turner et al., 1981; Newman and Jagoe, 1994). Among this group of metals that occur naturally as carbonate and hydroxide complexes in seawater are several of ecotoxicological interest, such as Cu(II), Pb(II) or Zn(II), and their speciation could be fixed by the presence of humic matter in the seawater. However, at normal metal and HA concentrations, only Cu, and perhaps Pb, are likely to be complexed in marine environments significantly (Turner et al., 1981; Morgan and Stumm, 1991).

Electroanalysis is regarded as the most suitable method for determining the speciation and bioavailability of elements and their complexes (Florence, 1986). Voltammetric methods such as Anodic Stripping Voltammetry (ASV) provide the most direct evidence in studies of metal speciation at trace levels, particularly in saline waters (Florence, 1982). Some similarities between the metal uptake through biological membranes and the electrodepositional step in voltammetric measurements have been established (Florence, 1986; Mota and Correia dos Santos, 1995), and the bioavailable fraction has been correlated with the concentration of the labile metal (Young et al., 1979; Florence, 1982; Deaver and Rodgers, 1996). Electrochemically labile metal is defined as the fraction of the total dissolved metal that can be deposited at the electrode surface under given conditions (Florence, 1983), and is usually attributed to the free metal ion, together with inor-

ganic and weak organic complexes. Nevertheless, in a buffered and chemically defined medium the free Cu ion activity is a constant fraction of the total inorganic concentration (Allen and Hansen, 1996; Lage et al., 1996).

Bioassays are a frequently used technique for assessing the biological effects of pollutants on aquatic organisms, and the high sensitivity of the early life stages of marine invertebrates offers a useful biological tool for laboratory and environmental studies in coastal ecosystems (His et al., 1999). Sea urchin larvae bioassays have thus been widely used to assess toxicity in seawater and marine sediment samples (e.g. Klöckner et al., 1985; Vashchenko and Zhdan, 1993; Kobayashi, 1995; Carr et al., 1996; Beiras et al., 2001). Nevertheless, little attention has been paid to the chemical aspects of toxicity testing, and some considerations regarding metal speciation should be taken into account (Batley et al., 1999).

In this work, the speciation of Cu in chemically defined seawater in the presence of humic acids was studied by means of Square Wave Anodic Stripping Voltammetry (SWASV), and toxicity tests with *P. lividus* embryos and larvae were performed in order to relate the measurements of Cu speciation to its bioavailability.

## 2. Material and methods

### 2.1. Reagents

Humic acid (HA) stock solutions (1 g/l) were prepared by dissolving the sodium salt (Aldrich Chemical Company) in a  $4 \times 10^{-3}$  M NaOH solution, and stored at 4 °C in dark to prevent photochemical ageing (Ortego and Benson, 1992). The carbon content of the HA salt was measured by using an elemental analyzer (Perkin–Elmer CNH 2400) and it was estimated to be 49.4% dry weight (the humidity of the salt was 20.7%). The standard copper solution (1.000 g/l) obtained from Panreac and the other reagents used were of analytical grade quality. All solutions were prepared with distilled water deionized by a Milli-Q<sup>50</sup> purification system (Millipore). All glassware and

polypropylene labware were soaked in 10% HNO<sub>3</sub> and rinsed in deionized water before use. Experimental solutions were prepared in chemically defined artificial seawater (ASW), according to the formulation shown in Table 1 (modified from Zarogian et al., 1969). Before each experiment, the pH and salinity of the samples were checked and found to be  $8.0 \pm 0.1$  and  $34 \pm 0.3$  ppt, respectively.

### 2.2. Cu–HA complexation model

Six sets of copper solutions in ASW were prepared at the following total metal concentrations: 30, 45, 60, 90, 120 and 180 µg/l. A series of increasing quantities of HA were added to each Cu concentration (12–14 additions per concentration) in order to obtain solutions containing decreasing amounts of labile Cu. The solutions were prepared in 25 ml volumetric flasks, transferred into polypropylene bottles, and kept for at least 24 h in the dark to allow equilibration of the complexation reaction (Ma et al., 1999).

SWASV analyses were carried out with a hanging mercury drop electrode, a Ag/AgCl reference and a Pt-rod auxiliary electrode held in a Metrohm 663 VA polarographic stand combined with a Eco-Chemie AutoLab PGSTAT10 potentiostat. After the solutions had been purged with N<sub>2</sub> for 5 min, the Cu was accumulated on a mercury drop of 0.52 mm<sup>2</sup> at –0.5 V for 20 s at the maximum stirring speed (3000 rpm) and 10 s

Table 1  
Composition of artificial seawater

Salt	Amount (g/l)
NaF	0.003
SrCl <sub>2</sub> ·6H <sub>2</sub> O	0.024
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> ·10H <sub>2</sub> O	0.0475
KBr	0.100
KCl	0.700
CaCl <sub>2</sub> ·2H <sub>2</sub> O	1.47
Na <sub>2</sub> SO <sub>4</sub>	4.00
MgCl <sub>2</sub> ·6H <sub>2</sub> O	10.78
NaCl	24.50
NaHCO <sub>3</sub>	0.200

of rest before the voltage scanning. The conditions of the square-wave (SW) voltammogram were at an initial SW potential of  $-0.5$  V, a SW amplitude of  $40$  mV, a SW frequency of  $40$  Hz and a scan increment of  $2$  mV. The voltammetric peaks appear at a potential between  $-160$  and  $-180$  mV without HA and between  $-160$  and  $-240$  mV with HA. Three polarograms were recorded for each solution and the mean value of the peak heights was calculated.

The data obtained were explained by adopting the simplest complexation model, assuming only one type of ligand and a reaction stoichiometry of 1:1:



The equilibrium system is described by the following equations (Buffle, 1984; Lund et al., 1990):

$$K' = \text{CuL}/\text{Cu}' \times \text{L}' \quad (1)$$

$$\text{Cu}_T = \text{Cu}' + \text{CuL} \quad (2)$$

$$\text{L} = \text{L}' + \text{CuL} \quad (3)$$

where  $\text{Cu}_T$  and  $\text{Cu}'$  are the total and labile copper concentrations,  $\text{L}$  and  $\text{L}'$  are the total and unbound ligand expressed as Cu binding concentration, and  $K'$  the conditional stability constant. From Eqs. (1)–(3):

$$\begin{aligned} K' &= \frac{\text{CuL}}{\text{Cu}'\text{L}'} = \frac{\text{Cu}_T - \text{Cu}'}{\text{Cu}'(\text{L} - \text{CuL})} = \frac{\text{Cu}_T - \text{Cu}'}{\text{Cu}'(\text{L} - \text{Cu}_T + \text{Cu}')} \\ &= \frac{\text{Cu}_T - \text{Cu}'}{\text{Cu}'\text{L} - \text{Cu}'\text{Cu}_T + (\text{Cu}')^2} \end{aligned}$$

We can thus express  $\text{Cu}'$  as a function of the total Cu and ligand concentrations:

$$\begin{aligned} K'\text{Cu}'\text{L} - K'\text{Cu}'\text{Cu}_T + K'(\text{Cu}')^2 &= \text{Cu}_T - \text{Cu}' \\ K'(\text{Cu}')^2 - K'\text{Cu}_T\text{Cu}' + K'\text{LCu}' + \text{Cu}' - \text{Cu}_T &= 0 \\ (\text{Cu}')^2 + \text{Cu}'(-\text{Cu}_T + \text{L} + 1/K') - \text{Cu}_T/K' &= 0 \end{aligned} \quad (4)$$

$$\text{Cu}' = \frac{-a + \sqrt{a^2 + 4\text{Cu}_T/K'}}{2} \quad (5)$$

where  $a = (-\text{Cu}_T + \text{L} + 1/K')$ .

### 2.3. Background metal content in the ASW

In order to study the background trace metal content derived from the reagents used to make up the ASW, a  $25$  ml sample from each stock of ASW was acidified up to pH 1.0 with  $\text{HNO}_3$ . Total metal content was measured by SWASV using an accumulation time of  $150$  s and an accumulation potential of  $-1.2$  V. Zn, Cd, Pb and Cu were measured during the same scan and the trace content was calculated by standard additions.

### 2.4. Biological material

Adult sea urchins (*Paracentrotus lividus*) were collected from a subtidal population at Canido ( $42^{\circ}11'36''\text{N}$ ,  $8^{\circ}49'30''\text{W}$ ), immediately transported dry to the laboratory in a cooler, kept in a seawater aquarium of  $200$  l capacity at  $15$  °C, and fed with *Ulva lactuca*. Gametes were obtained by dissection from a single pair of adults and their maturity checked under microscope (spherical eggs and mobile sperm). Eggs were transferred to a measuring cylinder containing  $100$  ml of ASW. A few microliters of dry sperm were collected directly from the gonad with a Pasteur pipette, added to the egg suspension and carefully stirred with a plunger to allow fertilisation. Five samples of  $50$  µl were taken to record fertilisation success (assessed by the percentage of eggs showing a fertilisation membrane) and egg density under the microscope. Four hundred eggs were delivered into  $20$  ml polypropylene vials. Five replicates per treatment and five ASW blanks were assayed for each experiment.

### 2.5. Bioassay tests

The vials were incubated at  $20 \pm 0.1$  °C for  $48$  h in the dark, to avoid photodegradation of the HA. This exposure time allows the complete development of embryos into pluteus larvae while minimising background mortality. After the incubation period, larvae were fixed with a few drops of  $40\%$  formalin. Two endpoints were recorded in each sample: the percentage of fully developed 4-arm pluteus larvae ( $n = 100$ ) and the mean larval length ( $n = 25$ ).

Table 2

Exposure medium for the Cu+HA experiments, and observed and predicted labile Cu concentrations

[Cu] tot (µg/l)	% Labile	[HA] (mg/l)	[Cu'] pred (µg/l)	[Cu'] obs (µg/l)
30	75	0.94	22.5	33.4
30	50	2.31	15	17.9
30	25	5.39	7.5	13.0
45	75	1.20	33.75	38.9
45	50	2.83	22.5	26.2
45	25	6.16	11.25	15.1
60	75	1.46	45	52.7
60	50	3.34	30	34.3
60	25	6.93	15	18.8
90	75	1.97	67.5	74.6
90	50	4.37	45	48.1
90	25	8.47	22.5	27.0
120	75	2.48	90	94.5
120	50	5.39	60	62.5
120	25	10.02	30	35.6
180	75	3.51	135	133.7
180	50	7.45	90	83.3
180	25	13.10	45	43.5

Three groups of experiments were performed in order to study the effect of speciation on Cu toxicity to larvae. First, the toxicity of HA at concentrations ranging from 0.039 to 40 mg/l was tested in two experiments. Secondly, the toxicity of Cu in the absence of HA was assayed at concentrations ranging from 10 to 180 µg/l in two other experiments. Thirdly, the toxicity of combinations of Cu and HA, including different proportions of labile Cu: 75, 50 and 25%, and six total Cu concentrations: 30, 45, 60, 90, 120, 180 µg/l, was tested in three experiments. The amount of HA added was calculated according to the complexation model developed previously (see Table 2). All the solutions were kept for 24 h in the dark at 20 °C before the bioassay in order to achieve equilibrium in the complexation reaction before embryo exposure. One sample of each solution was analyzed by SWASV to check the labile Cu concentrations on the day the bioassays were started.

## 2.6. Data analysis

The toxicity data were transformed using the Abbott correction to take into account the differences between stocks in experiments performed on different dates (His et al., 1999). The normality and

the homoscedasticity of the data were tested using the Kolmogorov–Smirnov test and the Levene test, respectively. The toxicity tests with HA were analyzed by ANOVA to detect the toxic effects on larval development and growth. The Dunnett a posteriori test was then applied to identify the toxic concentrations. The toxicity data with Cu only and with Cu+HA were fitted to a logistic model (Haanstra et al., 1985):

$$y = \frac{c}{1 + e^{b(X - a)}} \quad (6)$$

where  $c$  stands for growth in the control,  $a$  the median effective concentration ( $EC_{50}$ ) and  $b$  the slope of the dose–response curve. The toxicity parameters and the parameters of the complexation model (see below) were estimated by means of a non-linear fitting procedure (STATISTICA, StatSoft)

## 3. Results

### 3.1. Cu–HA complexation model

The effect of additions of HA on the anodic stripping voltammograms of the Cu solutions was

a decrease in the peak height, due to Cu complexation (Fig. 1). There was also an increase in the peak width and a slight shift in the peak potential towards more negative values. The background current also increased with HA concentration. The same results were observed disregarding the Cu concentrations under study.

After checking that the peak current was a linear function of Cu concentration, and assuming that the Cu–HA complexes are inert, and thus do not contribute to the peak height, the labile copper ( $\text{Cu}'$ ) was calculated as:

$$\text{Cu}' = \text{Cu}_T \times i_p / i_0$$

where  $\text{Cu}_T$  is the total Cu concentration in the solution,  $i_0$  the peak current of Cu in the solution without HA, and  $i_p$  the peak current of Cu obtained in the solution with HA.

Fig. 2a shows all the labile Cu concentrations measured at different concentrations of HA. We can fit these data to Eq. (5) and calculate the parameters of the complexation model,  $K'$  and  $L$ ,

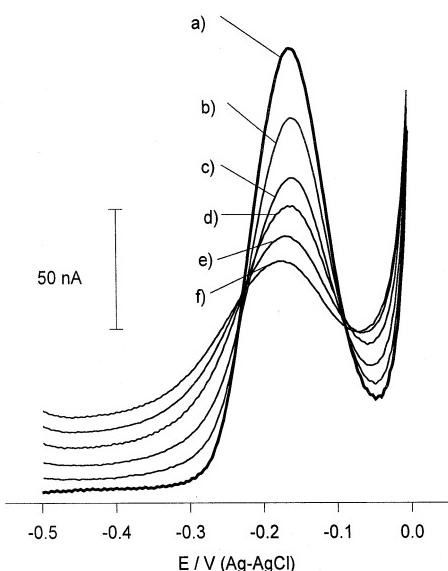


Fig. 1. Effect of the addition of increasing amounts of humic acids (HA) on the voltammogram of 120 µg/l of Cu in artificial seawater. (a) 0.0; (b) 2.0; (c) 4.0; (d) 6.0; (e) 8.0; and (f) 10.0 mg HA/l.

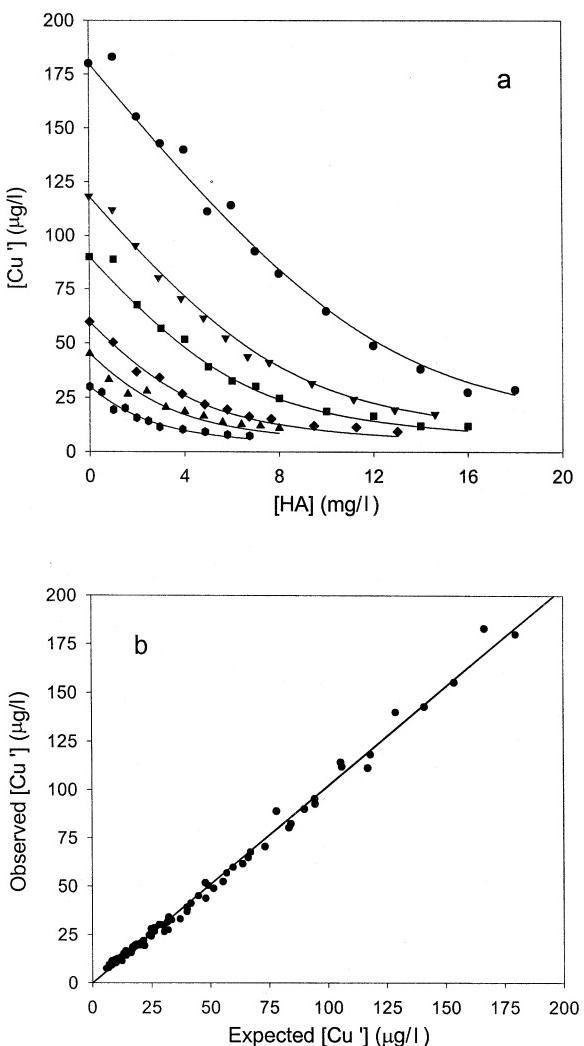


Fig. 2. (a) Concentrations of labile Cu, measured by SWASV, in six series of solutions with 30 ( $\blacktriangledown$ ), 45 ( $\triangle$ ), 60 ( $\blacklozenge$ ), 90 ( $\blacksquare$ ), 120 ( $\blacktriangledown$ ) and 180 ( $\bullet$ )  $\mu\text{g/l}$  of total Cu at increasing concentrations of HA. The lines show the concentrations predicted by a complexation model based on a single ligand and a 1:1 stoichiometry. (b) Predicted labile Cu concentrations vs. the observed labile Cu concentrations measured by SWASV. The line shows the linear regression model:  $y = -0.28 + 1.02x$ ;  $r^2 = 0.994$ .

by means of a non-linear fitting procedure. To relate  $L$  to the HA concentration, it is necessary to define the complexation capacity of the HA,  $N$  ( $\mu\text{mol Cu/g HA}$ ). The estimated parameters were

$\log K' = 6.53 \pm 0.05$ ,  $N = 230 \pm 7$  ( $\mu\text{mol Cu/g HA}$ ) and  $r^2 = 0.993$ . Fig. 2b shows the observed and predicted labile Cu values. The residues do not display any trend and the data fit well to a linear model with a slope of 1.020. This model is thus suitable for the general purposes of the present study. The HA concentrations required to give a percentage of labile Cu of 75, 50 and 25% for the planned experiments were therefore calculated from Eq. (4), and all the samples were analyzed before the exposure experiments to check the model. A high correlation between the nominal and observed concentrations of labile Cu was observed, with a slope of 1.033 and  $r^2$  of 0.975.

### 3.2. Background metal content in the artificial seawater

Metal concentrations were:  $[\text{Zn}] = 1.59 \pm 0.18 \mu\text{g/l}$ ;  $[\text{Cd}] = \text{not detected}$ ;  $[\text{Pb}] = 0.42 \pm 0.06 \mu\text{g/l}$  and  $[\text{Cu}] = 0.85 \pm 0.07 \mu\text{g/l}$ . These concentrations are much lower than the  $\text{EC}_{50}$  for *P. lividus* and they do not involve any risk to these organisms (Fernández and Beiras, 2001).

### 3.3. Bioassay tests

#### 3.3.1. Toxicity of the HA solutions

There was a small but significant decrease ( $7.7 \pm 5.1\%$ ) in embryogenesis success rates only at 40 mg/l, and there was no effect on the larval growth at any concentration. The HA concentrations used in the Cu + HA exposure experiments ranged from 0 to 12 mg/l. No toxicity is therefore attributable to the HA.

#### 3.3.2. Toxicity of the Cu solutions

The results of the two bioassays with Cu only were pooled and are shown in Fig. 3a and b. Fig. 3a shows the marked effect of Cu on embryotoxicity, while Fig. 3b shows a more gradual effect of Cu on larval growth. Growth inhibition has a lower threshold than the inhibition of embryogenesis, meaning that larval growth is a more sensitive endpoint for evaluating Cu toxicity. Both types of endpoint give good fits with a logistic model:

$$y = \frac{c}{1 + e^{b(X - a)}}$$

where  $b$  is the slope of the toxicity curve,  $a$  the  $\log_2 \text{EC}_{50}$ ,  $X$  the  $\log_2 [\text{Cu}]$ , and  $c$  the response in the controls ( $c = 100$ , because the Abbott correction was used to transform the toxicity data). The fitting parameters of these experiments are shown in Table 3, together with the parameters obtained with different models in the tests with Cu and HA (see below).

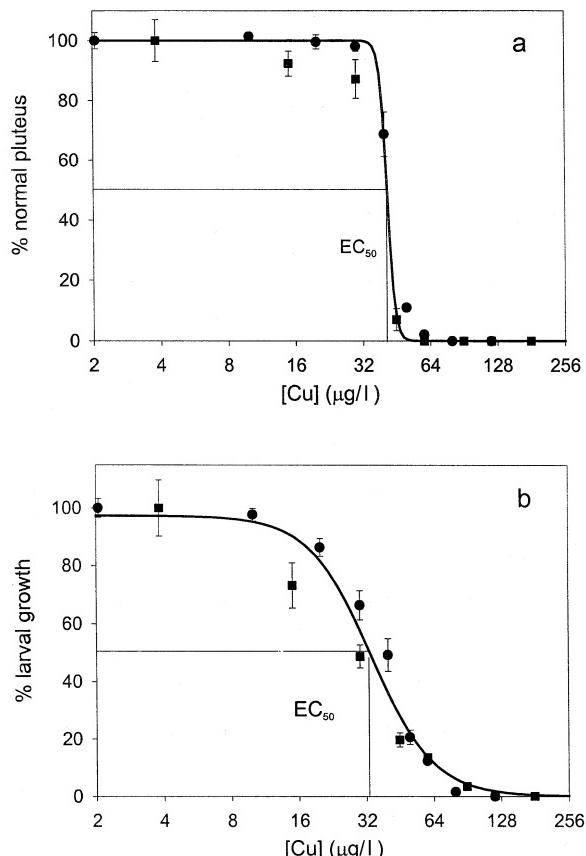


Fig. 3. Effect of Cu concentration on the success of embryogenesis (a) and larval growth (b) of *P. lividus*. The points show the mean percentage of normal pluteus larvae developed (a) and the mean percentage of larval growth compared to the controls (b) after 48 h of incubation at 20 °C for two different experiments (squares and circles). Error bars represent standard deviations. The solid line is the best logistic fit.

Table 3

Fitting parameters ( $\pm 95\%$  confidential intervals) of the toxicity curves for the individual experiments with exposures to Cu alone and to Cu+HA. [Cu<sub>T</sub>], total Cu concentration; [Cu'], labile Cu concentration; [Cu-HA], concentration of Cu-HA complexes

Model	<i>a</i>	<i>b</i>	<i>c</i>	<i>R</i> <sup>2</sup>	<i>F</i> (sign)
<i>Larval development</i>					
Cu only	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}_T] - a)}}$	$5.16 \pm 0.04$	$7.69 \pm 1.28$	—	97.89%
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}_T] - a)}}$	$5.74 \pm 0.12$	$2.56 \pm 0.84$	—	70.83%
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}_T] - a)}}$	$5.09 \pm 0.04$	$4.19 \pm 0.78$	—	92.13% 3.97 (0.0008)
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}'] - a + c \log_2[\text{Cu-HA}])}}$	$5.30 \pm 0.06$	$4.53 \pm 0.70$	$0.055 \pm 0.014$	94.56% 1.447 (0.20) ***
<i>Larval growth</i>					
Cu only	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}_T] - a)}}$	$4.71 \pm 0.10$	$1.57 \pm 0.20$	—	97.20%
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}_T] - a)}}$	$5.52 \pm 0.12$	$1.43 \pm 0.30$	—	73.91%
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}'] - a)}}$	$4.79 \pm 0.05$	$1.72 \pm 0.14$	—	95.12% 7.064 (0.0001)
Cu+HA	$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}'] - a + c \log_2[\text{Cu-HA}])}}$	$4.91 \pm 0.09$	$1.74 \pm 0.14$	$0.029 \pm 0.020$	95.40% 1.041 (0.46) **

### 3.3.3. Toxicity of the Cu+HA solutions

There is a remarkable decrease in the toxic effects of Cu solutions to *P. lividus* larvae with increasing amounts of HA. Fig. 4 shows the percentage of normal larval development and larval growth at 60 µg Cu/l at different percentages of labile fractions obtained by adding increasing amounts of HA. The same trend was observed in the remaining total Cu concentrations. In Figs. 5a and 6a the toxicity data are plotted against total copper concentrations for the Cu+HA experiments. Both figures make it clear that for a fixed amount of total Cu, toxicity decreases as HA concentrations increase (i.e. with lower amounts of the labile fraction). The parameters of the toxicity equation that fits the data are given in the second row of Table 3. The experimental data depart markedly from the toxicity curve obtained in the experiments with Cu alone (solid lines) and they cannot be fitted to a single plot. This means that there are factors other than the total Cu concentration that affect the toxicity in these experiments.

When toxicity is expressed as a function of labile Cu concentration rather than total Cu (Figs. 5b and 6b), the percentage of variability

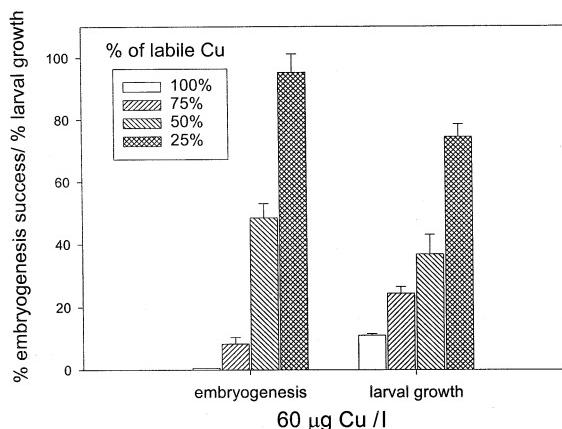


Fig. 4. Effect of HA concentration on the toxicity of 60 µg Cu/l to *P. lividus* larvae. The decreasing percentages of labile Cu were obtained by adding increasing amounts of HA. The columns represent the mean of the five replicates per treatment and the error bars the standard deviation values.

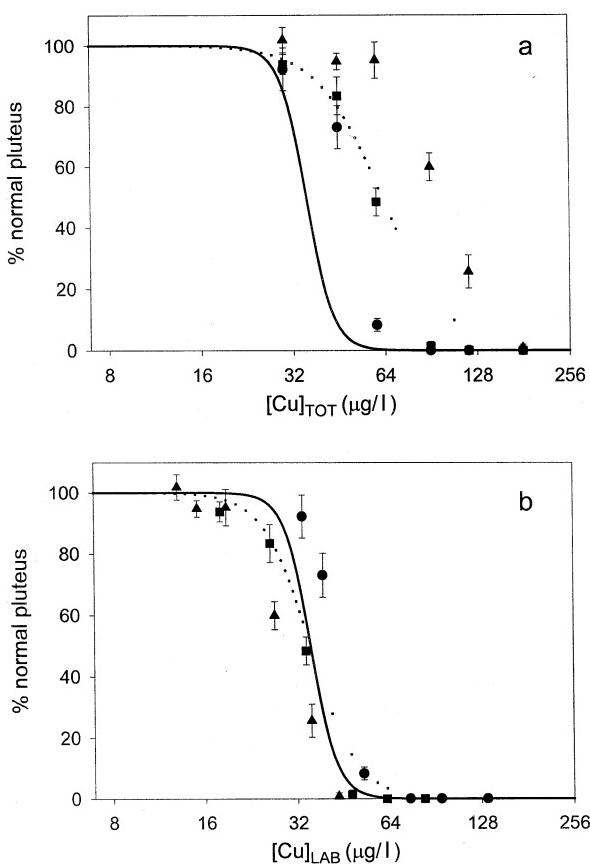


Fig. 5. Toxicity of Cu + HA to the embryogenesis of *P. lividus*. Percentage of embryogenesis success vs. the total copper concentration (a) and vs. the measured labile fraction by SWASV (b) for three experiments with Cu + HA exposures with different labile fractions: 25% (▲), 50% (■), and 75% (●) labile Cu. The dotted line represents the fitted curve to the data and the solid line the toxicity curve for the experiments with exposure to Cu alone. The data fit the toxicity curve much better when we express toxicity against the concentrations of labile Cu.

explained for the model improves from 70.8 to 92.1 in the experiments on embryogenesis success, and from 73.9 to 95.1 in the larval growth experiments. We can also see (Table 3) that the fitted parameters for this new model are not statistically different (except for the slope of embryogenesis success) from the parameters in the experiments with Cu alone. *F* test was performed to see whether the differences between observed and predicted values are significantly reduced when toxicity is expressed as a function of labile Cu rather

than total Cu. The *F* test shows a highly significant improvement ( $P = 0.0008$  and  $P < 0.0001$  for development and larval growth, respectively) in the predictive success of the new model. This means that labile Cu concentration is a better predictor of the toxicity of the Cu in the presence of HA than the total Cu concentration.

The data were also fitted to a third type of logistic model:

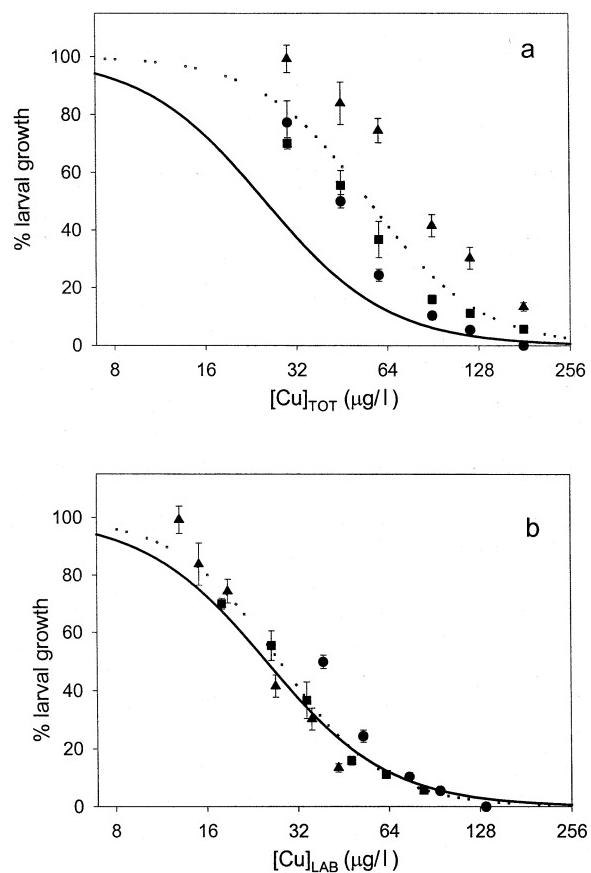


Fig. 6. Toxic effects of Cu + HA exposures on the larval growth of *P. lividus*. Percentage of larval growth vs. total copper concentration (a) and vs. the measured labile fraction by SWASV (b) for three experiments with Cu + HA exposures with different labile fractions: 25% (▲), 50% (■), and 75% (●) labile Cu. The dotted line represents the fitted curve to the data and the solid line the toxicity curve for the experiments with exposure to Cu alone. The data fit the toxicity curve much better when we express toxicity against the concentrations of labile Cu.

$$y = \frac{100}{1 + e^{b(\log_2[\text{Cu}] - a + c \log_2[\text{Cu} - \text{HA}])}}$$

where we have introduced a new parameter,  $c$ , to take into account any potential contribution of the toxicity of the Cu–HA complexes to the overall toxicity. With this new term there is a slight increase in the percentage of variability explained by the data. The values of  $c$  were rather small but statistically significant (see Table 3). However,  $F$  test showed that the decrease in the differences between observed and predicted values obtained using this new model compared with the one with only labile Cu were not significant ( $P = 0.20$  and  $P = 0.46$  for larval development and larval growth, respectively).

#### 4. Discussion

The addition of HA causes a decrease in the peak current on the voltammograms. This process is normally attributed to the formation of non-labile complexes between Cu and HA. There was also an increase in peak width and a slight shift in peak potentials towards more negative values. These phenomena are usually related to the partial lability of the complexes or to adsorptive processes on the electrode surface (Florence, 1982; Turner et al., 1987; Lund et al., 1990; Van Leeuwen, 1991; Fukushima et al., 1992; Labuda et al., 1994). The background current also increased with HA concentration, indicating the adsorption of HA on the electrode surface, due to their hydrophobic nature.

Metal–HA complexes, however, are usually assumed to be inert for the purpose of speciation studies (Srna et al., 1980; Turner et al., 1987; Van Leeuwen et al., 1989; Lund et al., 1990; Fukushima et al., 1992). Assuming that the Cu–HA complexes are non-labile, we developed a simple complexation model, with only one ligand type and a 1:1 stoichiometry that fits very well into the analytical data, explaining 99.3% of variability. When new solutions for the bioassays were prepared adding HA to the Cu according to this complexation model, measured labile Cu departed less than 20% from expected values. This procedure differs from normal titration, in which

the complexation capacity and the  $K'$  of the organic matter of the samples are calculated by adding known amounts of metal. In this kind of experiment, it is normal to obtain two different kinds of ligands, related to the carboxylic and the phenolic groups, respectively, by linearising Eq. (4). Nevertheless, good fits were also obtained with single-ligand models when non-linear fitting procedures were employed (Turner et al., 1987; Lund et al., 1990). Turner et al. (1987) also criticised the normal titration procedures and recommend the use of inverse titrations to test model systems, although these are not applicable to natural samples.

We have estimated a complexing capacity for 1 mg HA/l of 14.6 µg Cu/l, but need to bear in mind that the stability constant is relatively low, so that complexation is not complete. The values obtained in the present study,  $\log K' = 6.53$  and  $N = 230 \mu\text{mol Cu/g HA}$ , are comparable with those given by other authors for humic and fulvic acids, or for complexing organic matter from estuarine waters. Srna et al. (1980) found  $\log K'$  ranging from 6.4 to 6.7; Turner et al. (1987) found  $\log K'$  from 6.6 to 7.4 and  $N$  from 176 to 232 µmol Cu/g FA; Lund et al. (1990) obtained  $\log K'$  from 5.1 to 5.6 and  $N$  from 258 to 269 µmol Cu/g FA; finally Fukushima et al. (1992) reported  $\log K'_1 = 6.6$ ,  $\log K'_2 = 5.8$ , and  $N = 278 \mu\text{mol Cu/g HA}$ . We also need to bear in mind that the complexing parameters are operationally defined (Mota and Correia dos Santos, 1995) and that  $K'$  is not a thermodynamic constant but a conditional constant. These parameters are thus only applicable within the same range of total metal concentrations and within the conditions of the medium concerned. Moreover, the complexing parameters of humic matter are not the same as for a well-defined ligand. Humic substances are heterogeneous in nature, with different complexing sites, and the parameters given only describe a general stability constant. There exist more sophisticated models that take these polyfunctional properties into account by adding dispersion parameters (competitive Gaussian distribution model, Model V and NICA model (Perdue, 1998)), as well as the effect of electrostatic contributions to the binding strength. However, the

purposes of this work were not to describe the complexation mechanism but to predict the labile Cu fraction in seawater solutions; and the ASW medium used has a composition similar to most natural marine environments.

Toxicity tests showed no effects of HA on larval development and larval growth of *P. lividus*. There was only a small decrease in the percentage of larvae that developed normally at concentrations as high as 40 mg/l, but such concentrations are not realistic in the sea, even in coastal areas. The two Cu toxicity tests performed in this study provided consistent results for both endpoints: success of embryogenesis and larval growth. The EC<sub>50</sub> for both responses were also similar and very sensitive: 41.1 and 32.9 µg Cu/l, respectively. These values are comparable to the results obtained for success of embryogenesis by various authors for several species of sea urchin: in *P. lividus* 50–100 µg/l (Kobayashi, 1981, 48 h at 20 °C), ca. 31.8 µg/l (Pagano et al., 1986, 48 h at 20 °C), 47.6–63.5 µg/l (Warnau et al., 1996, 72 h at 18 °C); in *Arbacia punctulata* 33–44 µg/l (Carr, 1996, 48 h at 20 °C); in *Diadema setosum* 43 µg/l (Ramachandran et al., 1997, 48 h at 15 °C). Nevertheless, larval growth showed a much more gradual response to Cu toxicity, and effects could also be detected at lower concentrations. This is best illustrated by calculating the toxicity threshold expressed as EC<sub>05</sub>: 31.9 µg/l for success of embryogenesis and 12.2 µg/l for larval growth. These results highlight the extreme sensitivity of sea urchin bioassays to trace metals and support their use in studying the toxicity of environmental samples.

This study also demonstrates the high goodness of fit of toxicity data to the logistic model. Similarly, good results were obtained by other authors with data from different toxicants and organisms (Haanstra et al., 1985; Van der Geest et al., 1999). This model provides not only the EC<sub>50</sub>, but also the slope of the toxicity curve, thus offering a better description of the toxicity pattern of the pollutant. The logistic model also takes into account 0 and 100% values that cannot feed other models, and allows an easy calculation of the theoretical concentration that causes any given effect. For example, the EC<sub>05</sub> has been reported

by several authors to be a more reliable indicator of toxicity threshold than the No Observed Effect Concentration (NOEC) (Hoekstra and Van Ewijk, 1993, Anonymous 1998).

There was a clear decrease in Cu toxicity to *P. lividus* larvae in the presence of HA. This effect was observed with the HA concentrations used in this study, which can be considered realistic. A HA concentration of 3.2 mg/l was sufficient to reduce the embryotoxicity of 60 µg Cu/l by half, and 1.1 mg HA/l to increase larval growth at 45 µg Cu/l by a factor of 1.8. The same pattern was observed by several authors with natural organic matter or HA for many freshwater (Winner, 1984, 1985; Oikari et al., 1992; Porta and Ronco, 1993; Welsh et al., 1996) and marine organisms (Knezovich et al., 1981; Lores and Pennock, 1999). This means that in polluted estuaries, where Cu concentrations might otherwise reach values that are toxic to invertebrate larvae, HA may act as a protective barrier preventing the outbreak of toxicity events.

Labile Cu concentrations measured by SWASV provided accurate estimates of the toxicity of Cu in complexing environments. When we modelled toxicity as a function of labile rather than of total metal concentrations in the experiments with Cu + HA exposures, the explained variability improved significantly, and the fitting parameters were the same as those obtained in the Cu-only experiments. The logistic model that uses labile Cu as the explanatory variable could explain 92% of the variability in the success of embryogenesis and 95% of the variability in larval growth ( $n = 130$ ). The toxicity of seawater samples containing humic material will thus depend on labile Cu rather than the total Cu concentration. This fact has important consequences for the establishment of water quality criteria, because in estuarine and coastal waters large but highly variable amounts of humic substances are available. Nevertheless, it is important to take into account the fact that changes in pH, salinity and other parameters of natural waters may change the labile (bioavailable) fraction from the values predicted in the present complexation model.

The findings of this study generally support the FIAM and extend its application to the case of

natural organic matter in a marine environment. Previous experimental support of the FIAM in seawater was based on the use of well-characterized ligands which related measured biological responses to speciation values from theoretical models (Sunda and Guillard, 1976; Anderson and Morel, 1978; Zamuda and Sunda, 1982). Nevertheless, we could not find any work about the speciation and toxicity of Cu and HA in seawater. This is the first report, in our knowledge, on the correlation between measured metal speciation in the presence of humic material and the toxic effects on a marine organism. Studies about metal speciation using natural organic matter were limited to freshwater media. Most of them are fully consistent with FIAM (Sunda and Lewis, 1978; Meador, 1991; Deaver and Rodgers, 1996; Ma et al., 1999; Kim et al., 1999), but others indicated a slight toxic effect of the Cu–HA complexes (Giesy et al., 1983; Borgmann and Charlton, 1984; Erickson et al., 1996). Metal–HA complexes were also shown to be bioavailable for marine bivalves (George and Coombs, 1977; Pempkowiak et al., 1989; Kozuch and Pempkowiak, 1996).

In the present study, we found very weak evidence of additional toxicity attributable to the Cu–HA complexes. When a new parameter,  $c$ , is introduced into the toxicity model in order to take into account the concentration of the Cu–HA complexes (see Table 3), there was a slight increase in the explained variability of the model, but this is usual when the number of parameters in a model is raised. The new fitted parameter was extremely low (the Cu–HA complexes would be around 20–30 times less toxic than the labile Cu), but statistically significant. Nevertheless, the  $a$  and  $c$  parameters from Table 3 had a high degree of correlation, meaning that estimates of these two parameters are mutually dependent and that they can have some bias. Moreover, we could not find any statistical improvement in the goodness of fit of the new model vis-a-vis the model using labile Cu only. This was also supported by the lack of any visual trend in the residues of the toxicity explained as a function of the labile Cu alone. Finally, a  $c$  parameter small but significantly different than 0 might arise from the ab-

sence of a perfect correlation between the electrochemically labile Cu and the actual concentration of free Cu.

In conclusion, this study has demonstrated the protective effect of humic acids on the toxicity of copper and has provided a simple complexation model obtained via electrochemical measurements that quantitatively describes this effect with high accuracy. This high degree of concordance between the electrochemical and biological data indicates that ASV is a suitable method for estimating the bioavailable fraction of a metal in the marine environment with a minimum of manipulation of samples.

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